

Bcl₂-dependent antineoplastic effects of *Calotropis procera* root extract against canine mammary tumor cells

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Article Info	Abstract
Article history: Received: 09 January 2019 Accepted: 17 April 2019 Available online: 15 June 2021	There has been a prevailing trend in the application of herbal medicine as cancer therapeutics. <i>Calotropis procera</i> is an ayurvedic plant applied to ameliorate various illnesses. There is no report on the anti-tumor effects of the root of the plant on canine tumors, although it has been used for the treatment of various diseases in human medicine. The objective of the present study was to investigate the antitumor potential of ethanolic root extract of <i>C. procera</i> against canine mammary tumor cell line (CF41-Mg). MTT, western blot, and flow cytometry assays were carried out to evaluate the possible cytotoxicity and apoptosis induction of the extract. MTT results showed that the extract had a potent cytotoxic activity in a dose-dependent manner with an IC ₅₀ of 9.00 µg mL ⁻¹ . Based on the results of flow cytometry and western blotting, IC ₅₀ concentration of the extract induced significant apoptosis in the studied cell line, possibly through down-regulation of Bcl-2 expression. The results of the present study clearly indicated that the root extract of <i>C. procera</i> had promising anti-cancer activity and could be considered as a candidate for the treatment of mammary tumors.
Keywords: Apoptosis <i>Calotropis procera</i> Canine mammary tumor cell line Cytotoxicity Herbal medicine	

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Introduction

Nowadays, one of the major goals in human and animal cancer therapy is to identify the novel drugs with more promising outcomes and fewer side effects. Canine mammary tumors seem to mimic human breast cancers due to the range of proven similarities in the histological patterns and molecular levels. Therefore, the tumor could be used as a suitable model to study human breast cancer.¹ Canine mammary tumor cell line (CF41-Mg) is a mesenchymal-like canine mammary tumor cell line. Despite interesting features such as epithelial-mesenchymal transition (EMT) and lack of COX₂ expression there are just few studies on the cell line.^{2,3} EMT plays a key role in cancerous cell metastasis, cell invasion and increased resistance to anti-neoplastic drugs.^{3,4} Therefore, identifying the novel natural compounds with minimal side effects and the potential of inhibition of cancerous cells proliferation are of interest to many researchers. *Calotropis procera*, called giant milkweed, swallow-wort,

and apple of Sodom is a wild growing plant of *Asclepiadaceae* family and well known for its medicinal properties and has been used in traditional medicine for the treatment of leprosy, ulcers, tumors and diseases of skin, spleen, liver, and abdomen.⁵⁻¹² Furthermore, the plant is applied as an anthelmintic and expectorant.¹³ Most of the published data on the therapeutic properties of *C. procera* are related to the latex and aerial parts of the plant in human medicine.^{5,12-14} For instance, the leaves, flowers, and latex of the plant were proved to have anti-inflammatory (partly through inhibiting histamine, bradykinin, and prostaglandin E₂), analgesic, antioxidant, cytotoxic, and antibacterial activities.^{11,13,15-17} On the other hand, the root bark possesses anti-inflammatory and emetic properties and is recommended for treating chronic dyspepsia, flatulence, constipation, and anorexia.¹⁸ The methanolic and aqueous extracts prepared from the root of *Calotropis procera* have been reported to inhibit sub-acute inflammation in rodent models (via interruption of the arachidonic acid metabolism) and to combat free

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radical/metal ion-mediated oxidative damage (via free radical scavenging and metal ion chelating abilities), respectively.^{19,20} The plant is anthelmintic, the ashes act as an expectorant.

Due to the high prevalence of mammary tumors in dogs and lack of comprehensive study on the anti-tumor effect of *C. procera* in the treatment of canine mammary tumors, the present study was aimed to focus on the anti-tumor properties of the root-extract of *C. procera* on CF41-Mg cells.

Materials and Methods

Preparation of ethanolic extract of *C. procera*. The roots of *Calotropis procera* were collected from the southern areas of Iran and then identified and authenticated by the Department of Pharmacognosy, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran (Herbarium code: 1732). Contaminants were removed from the collected roots which were rinsed thoroughly with tap water. The roots were cut into small pieces, air-dried and ground into a powder using an electric-grinder. Details of the extraction process have been described previously.^{6,8} Briefly, the air-dried and powdered roots were treated with 96.00% ethanol (Merck Millipore Corporation, Darmstadt, Germany). The extract was then filtered, and low temperature and pressure rotary evaporator (Buchi AG, Flawil, Switzerland) was used to reduce the filtrate volume. The stock solution was then sterilized by filtration using sterile 0.22 µm pore size filter (Orange Scientific Co., Braine-l'Alleud, Belgium). The final concentrations of the extract were prepared as 1.00, 5.00, 10.00, and 20.00 µg mL⁻¹. The concentrations were selected in accordance with the previous in-vitro study²¹, and were confirmed using trypan blue experiment and morphological evaluation of the cells. The concentration of ethanol in these dilutions was restricted to no more than 0.50% (v/v) to minimize the potential effects of the solvent on the cell growth.

Cell culture. Canine mammary tumor cells, CF41-Mg (CRL-6232TM), were obtained from the Iranian cell bank of Pasteur Institute (Tehran, Iran) and were cultured according to the instructions of the American Type Culture Collection (ATCC[®]; Manassas, Virginia, USA). Briefly, cells were cultured as a single layer in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, USA) with 10.00% fetal bovine serum (Gibco), 60.00 IU mL⁻¹ penicillin G, 100 µg mL⁻¹ streptomycin, and 1.50 µg mL⁻¹ amphotericin B (Sigma Aldrich, St. Louis, USA). The culture medium was replaced every three days, and the cells were given passage using TrypLE[™] Select Enzyme (Gibco), after they had reached 80.00 - 90.00% confluency. The culture medium was removed and discarded from the flask and the cells were washed using sterile PBS. The pre-warmed corresponding enzyme (1.00 mL) was added to the flask

and incubated at room temperature for approximately 3 min. Following cellular detachment and addition of complete culture medium containing FBS (2.00 mL), the cell suspension was transferred to a conical tube and centrifuge then at 1,200 rpm for 5 min. Finally, the cell pellet was resuspended in a 5.00 mL of pre-warmed complete culture medium and poured into a flask.

Analysis of cell morphology. After sufficient cell growth in flasks (Orange Scientific Co.), CF41-Mg cells were disassociated using trypsin and were evenly added to 96 well plate (Orange Scientific Co.). Different concentrations of the extract were added to the wells after overnight incubation of the plate. Cellular morphology was compared between the treatment and control groups after 48 hr, using Motic[®] AE31 Elite inverted phase contrast microscope (Motic Co., Kowloon, Hong Kong). At the same time, cellular morphology was evaluated using Hematoxylin and Eosin (H&E) stained-salinized sterile slides by Leica microscope DM500 (Leica Microsystems Co., Wetzlar, Germany) and images were taken using ICC50 HD camera (Leica Microsystems Co.).

MTT assay. Cell viability was determined through MTT assay after addition of different concentrations of *C. procera* extract. The mitochondria of living cells can reduce Tetrazolium salt MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide) to formazan (1-[4, 5-dimethylthiazol-2-yl]-3, 5-diphenylformazan).^{22,23} Accordingly, cells with a density of 7.00 × 10³ cells per 200 µL were cultured in a 96 well plate (Orange Scientific Co.) and incubated overnight. Then, the old culture medium was replaced by 200 µL of a new culture medium containing the desired (1.00, 5.00, 10.00 and 20.00 µg mL⁻¹) concentrations of *C. procera* extract (five wells for each concentration). After 48 hr the medium containing the extract was removed and cell survival was analyzed using the MTT assay kit (Sigma Aldrich). To summarize, 100 µL of the MTT solution (5.00 mg mL⁻¹) was added to each of the wells and after 4 hr of incubation, the overlying fluid was disposed and 100 µL of dimethyl sulfoxide (DMSO; Sigma Aldrich) was added to the wells. The plate was shaken a few times and the optical densities (OD) of the wells were read at a wavelength of 490 nm using the ELISA reader device (ELX808; BioTek Instruments, Winooski, USA). After subtraction of the average medium background (OD_{Blank}) from average assay readings corresponding value (Corrected average OD) for each concentration (OD_{Sample}-OD_{Blank}) must then be divided by those of the control (OD_{Control}-OD_{Blank}) and multiplied by 100 to give cell viability (%). The formula was as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean corrected average OD of treated cells}}{\text{Mean corrected average OD of control cells}} \times 100$$

Finally, following drawing the dose-response curve, the required concentration for a 50.00% inhibition of cell survival (IC₅₀) was obtained graphically.

Apoptosis Assay. The quantitative analysis of apoptosis was carried out using ApoFlowEx® FITC kit (ExBio, Vestec, Czech Republic). The kit was designed to attach Annexin V to the phosphatidylserine which is transferred from the inner layer of the cell membrane to the outer layer when a cell is undergoing apoptosis.^{22,24-26} Cells were cultured in two flasks (Orange Scientific Co.) and the old culture medium was removed and replaced by a new medium containing ethanol and IC₅₀ concentration of *C. procera* extract, when the cell confluency of 80.00-90.00% was obtained. After 48 hr of incubation, the cells were disassociated using trypsin and were rinsed in ice cold PBS. In the next step, the cells were suspended in binding buffer and then propidium iodide (PI) and Annexin V. The suspension was vortexed gently and incubated for 15 min at room temperature in a dark environment. Annexin V, binding buffer and PI were removed and new binding buffer was added again to each tube. The cells were examined using the BD FACSCalibur flow cytometry device (BD Biosciences, San Jose, USA). To adjust the flow cytometer for evaluating the apoptosis unstained cells were used. Using these cells forward scatter (FSC)-side scatter (SSC) plot was drawn and subsequently cell debris was excluded. Next, based on the Annexin V (FL1) and PI (FL2) labeling properties, remained events were quantified in four quadrants of a dot plot. Viable cells excluded PI and were negative for Annexin V staining (lower left quadrant), whereas early and late apoptotic cells were Annexin V⁺/PI⁻ (lower right quadrant) and Annexin V⁺/PI⁺ (upper right quadrant), respectively.^{22,26} Likewise, necrotic cells were Annexin V⁻/PI⁺ (upper left quadrant). Accordingly, the percentage of stained cells in each quadrant and the total number of apoptotic cells (both early and late apoptosis) were quantified.

Western blotting analyses. Whole cell extract of the control and test groups (containing IC₅₀ concentration of *C. procera* extract) were prepared by the lysis of CF41-Mg cells using radio immunoprecipitation assay buffer (RIPA buffer; Sigma Aldrich) and centrifugation at 14,000 rpm for 20 min at 4.00 °C. Loading buffer (SDS, Glycerol, Tris, Bromophenol blue, and Dithiothreitol) was added after determination of protein concentration in each supernatant. In the next step, the mixture was heated at 95.00 °C for 5 min and then loaded onto a 10.00% denaturing polyacrylamide gel. Protein blotting was performed using nitrocellulose membrane (GE Healthcare Life Sciences, Amersham, UK) and blocking by incubating the membrane with 5.00% (w/v) skimmed milk (Merck Millipore) in Tris-Buffered Saline (TBS), at pH of 7.40, for 1 hr at room temperature and under continuous agitation. Immuno-labelling of the target proteins was carried out by anti-Bcl₂ (1:8000, BD Biosciences) and anti-β-actin (1:40000; Santa-Cruz Biotechnology Inc., Dallas, USA) antibodies as primary and horseradish peroxidase-conjugated goat anti-mouse IgG (1:40000, Sigma Aldrich)

as secondary antibodies. 5.00% (w/v) blocking buffer was used for antibody dilution. Then, immunodetection was recorded using ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories, Hercules, USA) and analyzed by Image Lab™ Software (version 3, Bio-Rad, California, USA).

Statistical analysis. The statistical differences between the treatments and the control groups were tested by one-way analysis of variance (ANOVA) followed by the Student's *t*-test using SPSS Software (version 16.0; IBM Corp., Armonk, USA). The Mean ± standard errors of means (SE) were calculated and the level of significance was considered as $p < 0.05$.

Results

CF41-Mg cells morphology. Light and invert microscopic evaluation of non-treated CF41-Mg cells revealed elongated and spindle-shaped cells with some malignancy criteria such as nuclear molding, numerous and abnormally prominent nucleoli and increased nuclear/cytoplasmic ratio (Fig. 1). Cellular morphology did not exhibit any significant change after treatment with ethanol (Fig. 1). However, 48 hr exposure of CF41-Mg cells to IC₅₀ concentration of *C. procera* extract caused significant changes in the appearance of apoptotic cells. The apoptotic cells lost their normal shapes and were changed to spherical cells with cytoplasmic shrinkage.

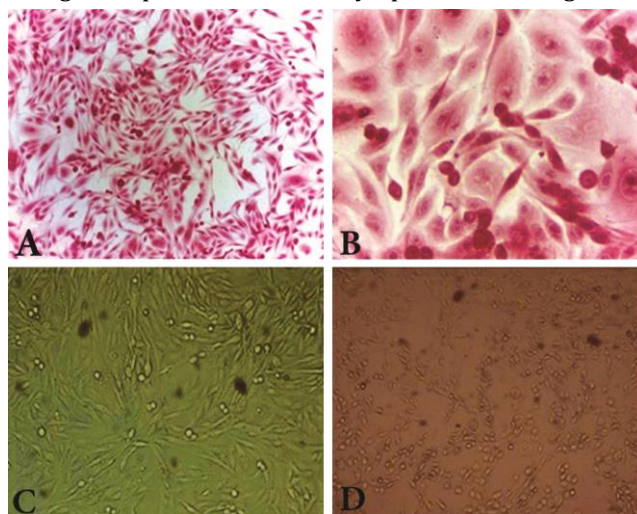


Fig. 1. Morphologic characterization of canine mammary tumor cells (CF41-Mg) before (A, B, and C) and after (D) treatment with *Calotropis procera* (IC₅₀ concentration). **A** and **B** represent H&E stained cellular morphology in magnifications of 100× and 400× respectively, while **C** and **D** exhibit invert microscope findings (100×).

Anti-proliferation effects of *C. procera* extract. The ethanolic root extract of *C. procera* was tested to assess its growth-inhibiting effect on CF41-Mg cell line. CF41-Mg cells were exposed to different doses of the extract (1.00, 5.00, 10.00 and 20.00 μg mL⁻¹) for 48 hr and the cell

viability was assessed by MTT assay. The mean percentages of cell survival at different doses of the extract were 78.45 ± 5.75 , 65.34 ± 7.09 , 45.91 ± 6.70 , and 27.65 ± 4.69 , respectively. The results suggested that the ethanolic extract treatment induced a dose-dependent inhibition of cell growth in CF41-Mg cells (Fig. 2). The IC_{50} value was determined as $9.00 \mu\text{g mL}^{-1}$.

Calotropis procera extract-induced apoptosis of CF41-Mg cells. Consistent with the anti-proliferative effect, the effect on apoptosis induction was observed after a 48 hr exposure to IC_{50} dose of the extract. As shown (Fig. 3), IC_{50} concentration of the extract decreased the number of viable cells and increased the number of apoptotic cells (as a sum of early and late apoptotic cells), significantly ($p < 0.001$).

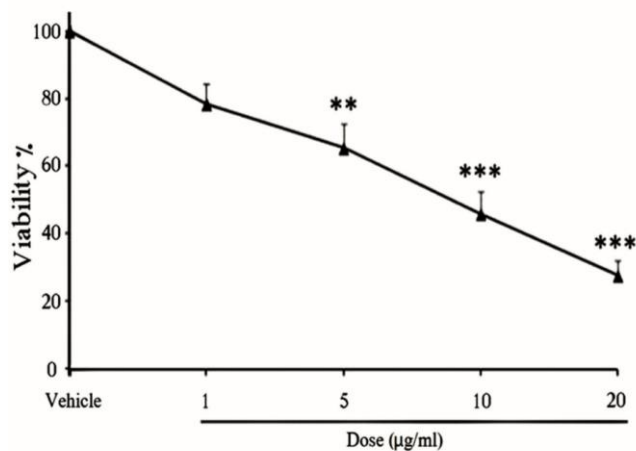


Fig. 2. Cell viability (%) after incubation with different concentrations of *Calotropis procera*. Data are expressed as the percentage of inhibition compared to control (mean \pm SE, $n = 8$). ** $p < 0.01$, *** $p < 0.001$.

Immunoblotting results. Western blotting for detection of Bcl2 expression in CF41-Mg cells showed that Bcl2 expression was significantly different between the control and test groups ($p < 0.05$). The exposure of CF41-Mg cells to IC_{50} concentration of *C. procera* extract caused suppression in Bcl2 expression (Fig. 4).

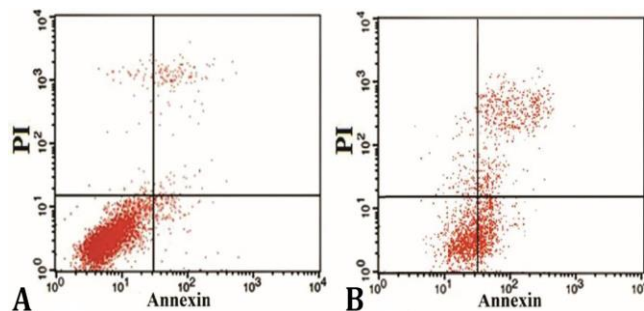


Fig. 3. Effects of IC_{50} concentration of *Calotropis procera* extract ($9.00 \mu\text{g mL}^{-1}$) on CF41-Mg cells apoptosis. Flow cytometric analysis of vehicle (ethanol, A) and extract-treated cells (B) stained with Annexin V and propidium iodide (PI) are presented in C and D. Mean values for five experiments \pm SE are illustrated. *** $p < 0.001$.

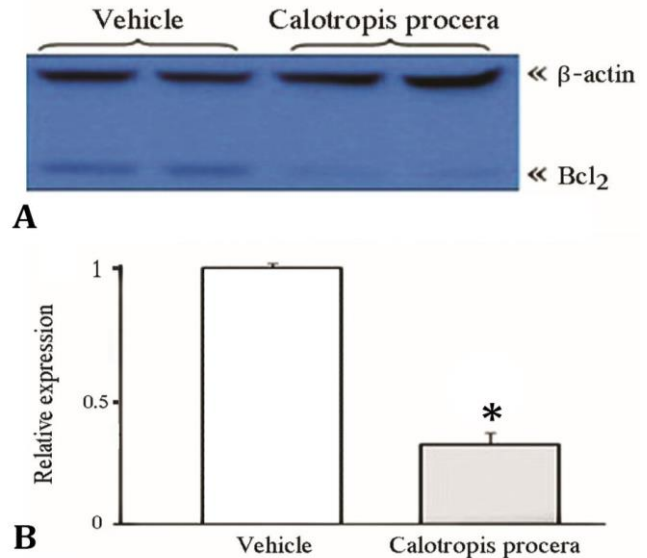
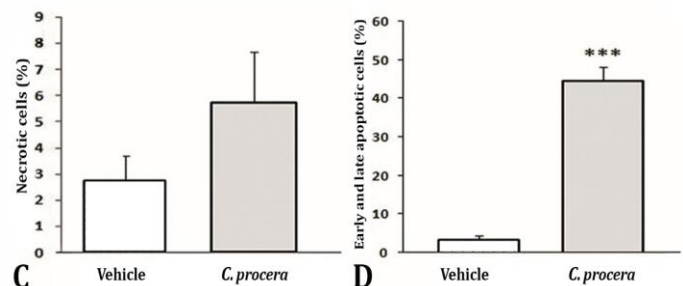


Fig. 4. Bcl2 expressions before and after treatment with *Calotropis procera* extract (IC_{50} concentration). **A)** Western blotting analysis of Bcl2 expression in CF41-Mg cells after treatments with ethanol as a vehicle (Control group) and IC_{50} concentration of root extract of *Calotropis procera* ($9.00 \mu\text{g mL}^{-1}$). **B)** Statistical analysis of Bcl2 expression in test groups compared to control groups. Relative protein levels quantified with the help of β -actin (loading control). The mean \pm SE are illustrated. * $p < 0.05$.

Discussion

Herbal medicine has mostly been identified by various experimental and clinical studies as a promising approach in cancer therapy.^{5,8,12} *Calotropis procera* is an ayurvedic plant containing various phytoconstituents (e.g. alkaloids, flavonoids and sterols) applied to ameliorate various illnesses.^{27,28} The extracts from different parts of the plant have been reported to have significant therapeutic effects, however, to the best of our knowledge no study has been conducted on the effects of *C. procera* in veterinary medicine. Therefore, the present study investigated the *in vitro* cytotoxic and apoptotic effects of ethanolic root extract of the plant on canine mammary tumor cell line (CF41-Mg). The MTT assay results indicated that the



ethanolic root extract of *C. procera* decreased viability of CF41-Mg cells in a dose-dependent manner following 48 hr exposure. Significant difference in cell viability was seen after incubating the cells with concentrations equal to or greater than 5.00 $\mu\text{g mL}^{-1}$. According to the dose-response curve (Fig. 2), IC_{50} concentration was calculated as 9.00 $\mu\text{g mL}^{-1}$. In a previous study,²¹ it was reported that different doses (1.00, 5.00, 10.00, and 25.00 $\mu\text{g mL}^{-1}$) of the root extract of *C. procera* possessed anti-tumor potential. They reported that Hep2 treated cells with the acetate extract (10.00 $\mu\text{g mL}^{-1}$) of *C. procera* showed the strongest cytotoxic effect (96.30%) following 48 hr exposure, whereas methanolic and hexane extracts exhibited cytotoxicity of 72.70 and 60.50%, respectively. Also, the root extracts caused apoptosis through cell cycle arrest at the S phase and prevented Hep2 cells from entering G2/M phase.²¹ In another study, strong cytotoxic effect of the root extract of *C. procera* on COLO 320 tumor cells was observed.²⁹ To elucidate growth inhibition mechanisms by the root extract of *C. procera*, apoptosis assay was performed in our study. Significant apoptosis induction ($p < 0.001$) by IC_{50} concentration of the root extract (Fig. 3) caused prominent morphological changes in the test group and increased events in the lower and upper right quadrants (indicated as percentages of early and late apoptotic cells). In contrast to spindle-shaped control cells, most CF41-Mg cells in the test group lost their integrity showing spherical morphology (Fig. 1). The increase in both early and late apoptotic activity indicated that regulation in apoptosis was at least partially responsible for cytotoxic effect of the extract.²² Apoptosis induction is a favorable property for anti-tumor compounds.^{24,25} Several mechanisms have been described for the induction of apoptosis by various compounds and the mitochondrial pathway seems to be the most common apoptotic mechanism. Cytotoxic stress leads to expression of the proteins of the Bcl family which acts independently or in combination.^{25,30} The degree of apoptosis was correlated with the level of Bcl-2 expression and main anti-apoptotic protein.³⁰ In order to identify the mechanism of apoptotic effect of *C. procera* extract, western blotting was performed. According to the obtained results (Fig. 4), the expression of Bcl₂ protein was down-regulated in the test group compared to the control group. Based on the few studies on anti-tumor effects of the root extract of *C. procera*, it seems that *C. procera* protein (CP-P) and UNBS1450 01 are the prominent effective anti-tumor compounds in this extract.^{29,31-33} UNBS1450 01 is a cardiotonic steroid from *C. procera* with anti-tumor activity. The compound has been proven to be a potent sodium pump inhibitor that can induce anti-proliferation effects and cell death. The mechanisms for these activities include repression of nuclear factor-kappa B (NF-kB) activation, down-regulation of C-Myc in cancerous cells, disorganization of the actin cytoskeleton after binding to

the sodium pump at the cellular membrane and induction of autophagy-related cell death.^{29,32,34} Samy *et al.* proposed that CP-P isolated from the root bark showed cytotoxic and apoptotic effects on breast cancer cells.³¹ The proposed mechanisms were suppression of NF-kB activation and down-regulation of NF-kB-regulated gene products (Cyclin D1 and Bcl-2) expression.^{31,35} Although this study indicated that down-regulation of Bcl₂ is one of the engaged mechanisms in anti-tumor activity of ethanolic root extract of *C. procera*, however, more studies are needed to recognize the other possible anti-tumor mechanisms of the extract.

Based on the results of the present study it could be concluded that *C. procera* could be a source of anti-tumor compounds and could be used in the treatment of canine mammary tumors in combination with the routine therapies. Clinical trials need to be performed to confirm the present results.

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Conflicts of interest

The authors declare no conflict of interest.

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